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SITE OF ACTION OF ANTIDIURETIC HORMONE ON MAMMALIAN NEPHRONS. (U)

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Final Report on
OMP N000 14-75-C-0166 - 1968-1979
SITE OF ACTION OF ANTIDIURETIC
HORMONE ON MAMMALIAN NEPHRONS

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This report summarizes some of the research findings supported in whole or in part by the Office of Naval Research Contract N00014-75-C-0166, "Site of Action of ADH on Mammalian Nephrons." The duration of the grant was 1968-1979. The initial support was for the development of a study to determine the effect of antidiuretic hormone (ADH) on kidney salt and water absorption utilizing the stop-flow technique to localize which areas of the nephron were affected by ADH and also how unidirectional Na and H₂O fluxes across the nephron were altered in order to study the mechanisms of ADH action. Following the conclusion of these experiments and based on the results, the research was broadened to include the mechanisms of action of other hormones or drugs or neurotransmitters on kidney and gut salt and water absorption and in particular whether these agents act through direct cellular effects or through physical forces exerted through the cardiovascular system.

There are two basic mechanisms by which salt, water and small organic solutes cross epithelial tissues, such as the gut and kidney, from the lumen into the blood. One mechanism is by transcellular transport across the luminal mucosal or apical cell membrane and then through the basolateral or serosal cell membrane. Solutes are transported by membrane carrier molecule pumps which transport the solutes by facilitated or active transport. A second mechanism by which many of the same solutes cross the membrane is through passive, paracellular movement through the tight junctions and the lateral intercellular spaces between the epithelial cells. Peptide hormones can directly affect transcellular movement by binding to the cell membranes at receptor sites and modulating intracellular events which increase or decrease pump activity. These same hormones could have cardiovascular effects which modify transcellular pumping by altering the delivery of O₂ to the cells for support of transport energy requirements. Passive, paracellular transport

can be altered by hormones acting through cardiovascular effects by changing blood flow and thus the degree of "washout" of absorbed molecules from the epithelium and by changing capillary and tissue pressures resulting in a change in the magnitude of ultrafiltration across the capillaries and epithelium. The research supported by this grant investigated several aspects of the above mechanisms.

This report is organized as follows. The first section summarizes findings of key phases of the research project. The second section lists dissertations and publications supported in whole or in part by the grant. The third section contains copies of the publications. Copies of some of the dissertations have already been received by ONR.

SECTION I - SUMMARIES OF RESEARCH FINDINGS

1. STOP-FLOW EXPERIMENTS

The mammalian kidney is composed of numerous unit nephrons which are themselves divided into anatomically and functionally distinct regions along their length. Antidiuretic hormone increases H_2O absorption in the distal nephron by increasing passive H_2O permeability in this region and thus allows H_2O conservation. There are additional effects on H_2O reabsorption which are exerted, in part, through effects on urea permeability, salt reabsorption, blood flow to different regions of the kidney and interactions among these different mechanisms. Individual segments of the nephron can be studied by micropuncture or microperfusion techniques but technical problems and the removal of the interactions among some of the processes involved in the final urine formation may reduce the applicability of these findings. In order to circumvent these possible problems, an in vivo method was devised utilizing

the stop-flow technique in which unidirectional Na and H₂O fluxes could also be measured. Measuring unidirectional fluxes allowed the possibility that effects on net transport could be assigned to carrier mediated transcellular transport (absorptive or lumen-to-blood flux) or to passive paracellular transport (secretory or blood-to-lumen flux). Also, because ADH is a small filterable molecule, it will be present at both the luminal and contraluminal surfaces of the nephron. The stop-flow technique allows ADH to be restricted to the contraluminal surface so that only responses to serosal exposure could be studied.

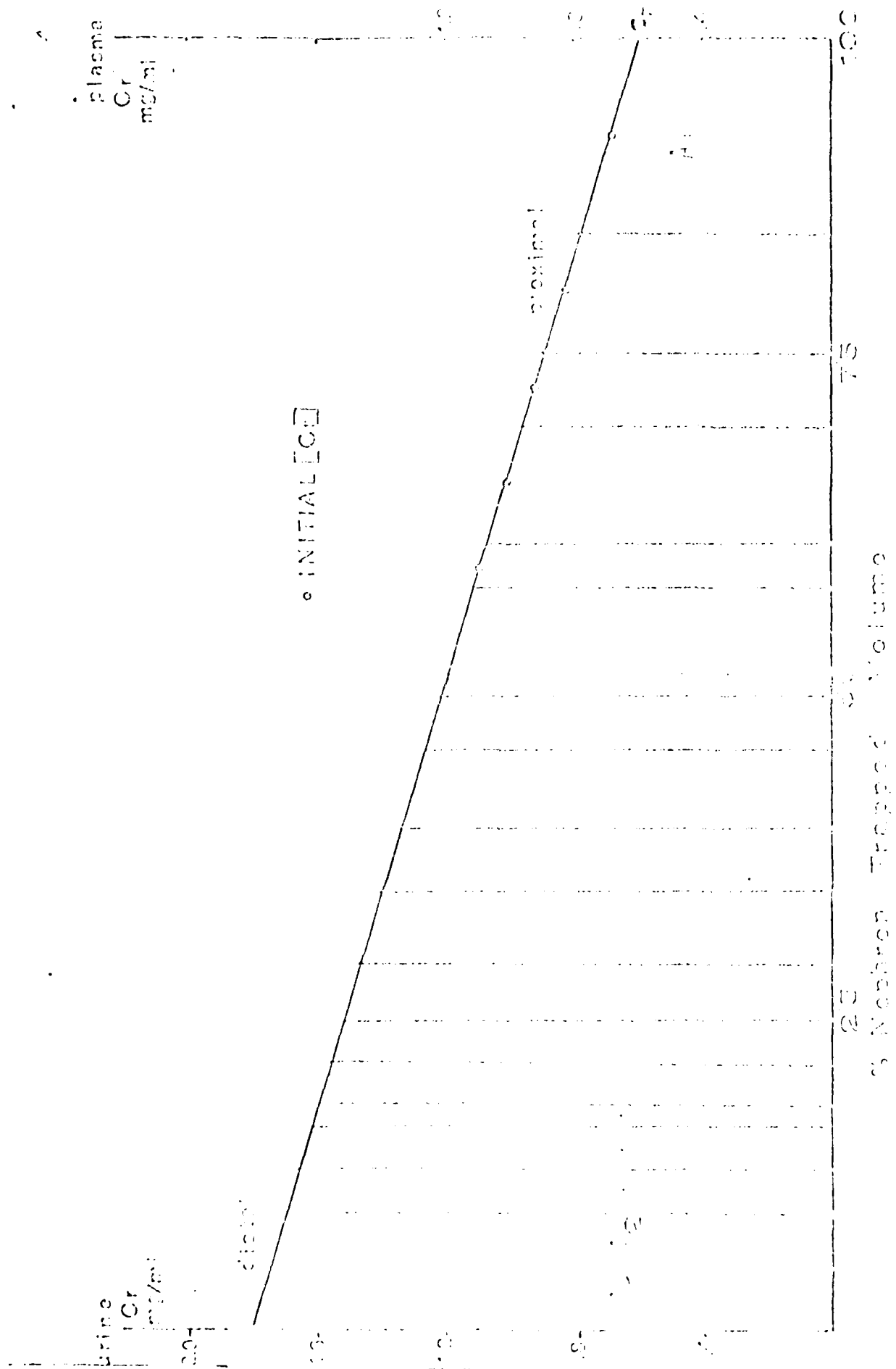
Dogs, anesthetized with Nembutal, were utilized as experimental animals. Both ureters were cannulated for urine sampling and the femoral arteries and veins cannulated for obtaining blood pressures, plasma samples and infusions. An infusion of 4% or 20% mannitol in isotonic saline containing creatinine, for measurement of glomerular filtration rate (GFR) and para-aminohippuric acid (PAH), for measurement of effective renal plasma flow was begun. After a high, stable urine flow was achieved urine and plasma samples were obtained. At the beginning of the stop-flow periods, ureteral pressure was raised to that of mean blood pressure by means of a mercury manometer, through a small reservoir containing the flowing urine. The rapid rise in pressure substantially reduced replacement filtration, i.e., the entry of glomerular filtrate during nephron absorption in the stop-flow period. After ureteral pressure had stabilized (15 sec), a solution (10 ml) of isotonic saline containing ³H₂O, ¹⁴C-inulin and ²²Na was infused IV at an exponentially decreasing rate, using mixing chambers, in order to create a step-change in isotope levels in the blood which did not vary by more than 8-25% during the 4 min period. Subsequent periods had twice the previous amount of isotope infused. ADH (20 or 100 mU/kg) was injected either simultaneously with the

isotope infusion to limit ADH primarily to the contraluminal nephron surface or two-minutes before to allow ADH exposure at both the luminal and contraluminal surface. Control experiments did not have ADH injections. At the end of the 4 min stop-flow period sequential urine samples were collected through the ureteral cannula.

The volume of each urine sample was determined gravimetrically. Cr and PAH were determined by standard colorimetric techniques. Na and K concentrations were determined by flame photometry and Cl by coulombmetric titration. $^3\text{H}_2\text{O}$, ^{14}C -inulin and ^{22}Na were determined by liquid scintillation spectroscopy.

Using the assumption that the concentrations of Cr, Na, K and Cl were linear functions, ranging between the plasma and final urine values, of the free-flow volume of tubular urine during an osmotic diuresis, the initial Cr concentration could be calculated by $C_2^2 = -C_1^2 A/A_T + C_n^2 A/A_T + C_1^2$ where C_1 is the final urine [Cr] during free-flow just before the stop-flow period; C_n is plasma [Cr]; C_2 is the initial tubular urine [Cr] just before stop flow; A_T represents the total amount of Cr in the stop-flow samples and A represents the distal-to-proximal summation of Cr in sequential stop-flow samples. The appearance of ^{14}C -inulin derived from the post-stop-flow IV infusion signalled the appearance of fresh glomerular filtrate and thus the end of the stop-flow samples. High [PAH] indicated proximal nephrons. The initial Na, K and Cl could be calculated for each stop-flow sample from the initial volumes and the free-flow urine and plasma concentrations. A sample graph of initial [Cr] is shown in Fig. 1. The derivation of the above equation was given in a previous submission (T. Burke, PhD dissertation). Unidirectional Na and H_2O fluxes were calculated from a modification of the method of Berger and Steele (J. Gen. Physiol. 41, 1135, 1958).

Figure 1. A computer integrated graph used to determine the initial concentrations of creatinine. C_1 and C_n are the free-flow urine and plasma concentrations of creatinine respectively. The black circles represent the computed initial concentrations of creatinine in each urine sample at the moment of stop-flow. Each vertical bar A_1, A_2, \dots, A_j represents the total initial amount of creatinine in that trapped stop-flow sample. Summing $A_1 + A_2 + \dots + A_j$ equals the total initial amount of creatinine trapped in the stop-flow kidney. Water is reabsorbed from the filtrate as it passes through the nephron. Therefore, the urine creatinine concentration (C_1) will be higher than the plasma creatinine concentration (C_n). The total volume of filtrate within the kidney is set at 100%. Less trapped volume is present in the distal regions compared to a larger trapped volume in the more proximal regions.



Partial results from one series (4% mannitol infusion, 100 mU ADH/kg) are shown in Tables 1-4. The results indicate that there is distal secretion of K^+ and proximal reabsorption (Table 1) in agreement with data of others. ADH present at the blood (contraluminal) side or at both sides (luminal and contraluminal) increases K secretion both proximally and distally. Sample "O" represents the stop-flow sample which has the distal Na concentration minimum. Samples are numbered sequentially distal (-) and proximal (+) to this sample so that similar regions of the nephrons can be matched. The division of samples into proximal and distal regions is based on [PAH]. ADH present at both sides of the nephron increased distal K secretion above that present when ADH was present only at the blood side as determined by analysis of variance (ANOVA) for individual values.

ADH at the blood or both blood and luminal surfaces increased net H_2O reabsorption distally but had no significant effect proximally (Table 2) in agreement with the results of others. However, the increase in distal net H_2O reabsorption was due to a decrease in H_2O entry into the lumen when ADH was present at the blood side only (Table 3) but an increase in H_2O leaving the lumen when ADH was present at both nephron surfaces (Table 4). Also, the lack of ADH effect on net H_2O reabsorption proximally was due to significant and similar increases in both the entry and exit of H_2O (thus the difference remained constant) with no difference between the effects of ADH when at the blood surface or both surfaces of the nephron (Tables 3, 4). Net and unidirectional Na fluxes (not shown) paralleled the H_2O fluxes.

The above results were not inconsistent with generally accepted findings, based on studies of individual nephrons, that ADH increases net H_2O reabsorption across the distal nephron and that its effects are exerted only from the blood side. However, certain of the other findings in these

TABLE 1

NET POTASSIUM FLUX AND THE
NEPHRONS OF THE LGC
($\mu\text{Eq/ml}$)

DISTAL	CONTROL	BLOOD	BOTH
-3	1.57 ± 0.40	1.03 ± 0.55	2.91 ± 0.74
-2	0.80 ± 0.48	1.39 ± 0.49	2.67 ± 0.95
-1	$-1.20^* \pm 0.86$	1.51 ± 0.73	3.34 ± 1.50
0	-2.71 ± 0.63	0.43 ± 0.32	0.93 ± 1.72
+1	-4.03 ± 0.74	-0.83 ± 0.52	-0.69 ± 1.26
+2	-4.00 ± 0.38	-1.16 ± 0.55	-1.05 ± 1.06
TOTALS	-13.27	-2.92	8.36
ANOV**	Control vs blood + both. Blood vs both.		$p < .01$ $p < .05$

PROXIMAL	CONTROL	BLOOD	BOTH
+3	-2.87 ± 0.65	-0.45 ± 0.58	-0.72 ± 1.27
+4	-2.35 ± 0.99	2.04 ± 0.79	-0.54 ± 1.14
+5	-1.63 ± 0.79	1.95 ± 0.36	0.45 ± 1.21
+6	-1.52 ± 1.20	0.97 ± 0.71	0.31 ± 1.20
+7	-3.37 ± 1.38	0.60 ± 0.95	-0.36 ± 1.12
TOTALS	-11.72	5.12	-0.83
ANOV**	Control vs blood + both. Blood vs both.		$p < .01$ $p = \text{NS}^{***}$

All values are mean \pm SE.

* Negative values represent potassium net flux out of the lumen.

** Complete analysis of variance on Table XX.

*** Not significant (NS).

TABLE 6.

NET WATER FLUX ACROSS THE NEPHRONS
OF THE LDC
(ml/4 min)

DISTAL	CONTROL	BLOOD	BOTH
-3	0.06 \pm 0.03	0.05 \pm 0.03	0.03 \pm 0.16
-2	0.23 \pm 0.13	0.23 \pm 0.09	0.28 \pm 0.16
-1	0.27 \pm 0.09	0.40 \pm 0.11	0.41 \pm 0.23
0	0.35 \pm 0.07	0.61 \pm 0.16	0.62 \pm 0.26
+1	0.51 \pm 0.07	0.66 \pm 0.17	0.71 \pm 0.28
+2	0.50 \pm 0.09	0.71 \pm 0.15	0.66 \pm 0.19
<u>TOTALS</u>	1.32	2.66	2.71
ANOV*	Control vs blood + both. Blood + both.		p<.01 p=NS**

<u>PROXIMAL</u>			
+3	0.61 \pm 0.13	0.73 \pm 0.22	0.72 \pm 0.20
+4	0.81 \pm 0.15	0.91 \pm 0.27	0.88 \pm 0.21
+5	0.99 \pm 0.15	1.06 \pm 0.25	0.93 \pm 0.23
+6	1.15 \pm 0.23	1.52 \pm 0.38	1.03 \pm 0.18
+7	1.64 \pm 0.39	2.23 \pm 0.85	1.40 \pm 0.29
<u>TOTALS</u>	5.20	6.45	4.96
ANOV*	Control vs blood + both. Blood vs both.		p=NS p=NS

All values are mean \pm SE.

* Complete analysis of variance on Table XX.

** Not significant (NS).

TABLE 2

WATER FLUX INTO THE LUMEN
OF THE DOG NEPHRON
(ml/min)

DISTAL	CONTROL	BLOOD	BOTH
-3	0.05 \pm 0.01	0.04 \pm 0.01	0.06 \pm 0.02
-2	0.08 \pm 0.02	0.03 \pm 0.02	0.09 \pm 0.03
-1	0.12 \pm 0.03	0.05 \pm 0.03	0.11 \pm 0.04
0	0.15 \pm 0.03	0.08 \pm 0.05	0.13 \pm 0.05
+1	0.16 \pm 0.03	0.12 \pm 0.05	0.16 \pm 0.05
+2	0.17 \pm 0.05	0.15 \pm 0.09	0.23 \pm 0.06
<u>TOTALS</u>	0.73	0.47	0.78
ANOV*	Control vs blood + both. Blood vs both.		p=NS** p<0.01

<u>PROXIMAL</u>			
+3	0.15 \pm 0.05	0.19 \pm 0.11	0.29 \pm 0.07
+4	0.17 \pm 0.06	0.24 \pm 0.11	0.36 \pm 0.08
+5	0.18 \pm 0.07	0.27 \pm 0.10	0.32 \pm 0.05
+6	0.25 \pm 0.09	0.41 \pm 0.15	0.35 \pm 0.06
+7	0.31 \pm 0.13	0.60 \pm 0.21	0.57 \pm 0.09
<u>TOTALS</u>	1.06	1.71	1.91
ANOV*	Control vs both + blood. Blood vs both.		p<0.01 p=NS**

All values are mean \pm SE.

* Complete analysis of variance on Table XX.

** Not significant (NS).

TABLE 4

WATER FLUX OUT OF THE LUMEN
OF THE DDB IMPIRION
(ml/min)

DISTAL	CONTROL	BLOOD	BOTH
-3	0.06 \pm 0.03	0.05 \pm 0.03	0.07 \pm 0.06
-2	0.14 \pm 0.05	0.09 \pm 0.02	0.14 \pm 0.05
-1	0.19 \pm 0.06	0.15 \pm 0.02	0.21 \pm 0.05
0	0.23 \pm 0.04	0.23 \pm 0.05	0.29 \pm 0.07
+1	0.26 \pm 0.05	0.19 \pm 0.07	0.34 \pm 0.07
+2	0.30 \pm 0.06	0.33 \pm 0.09	0.39 \pm 0.05
<u>TOTALS</u>	1.18	1.14	1.44
ANCOV*	Control vs blood + both. Blood vs both.		p=NS** p=NS

<u>PROXIMAL</u>			
+3	0.30 \pm 0.07	0.37 \pm 0.11	0.47 \pm 0.04
+4	0.37 \pm 0.08	0.46 \pm 0.13	0.60 \pm 0.03
+5	0.42 \pm 0.09	0.53 \pm 0.11	0.59 \pm 0.05
+6	0.54 \pm 0.14	0.79 \pm 0.19	0.64 \pm 0.06
+7	0.72 \pm 0.22	1.15 \pm 0.39	0.97 \pm 0.12
<u>TOTALS</u>	2.35	3.30	3.27
ANCOV*	Control vs blood + both. Blood vs both.		p<.01 p=NS**

All values are mean \pm SE.

* Complete analysis of variance on Table XX.

** Not significant (NS).

results did not necessarily fit with the expected increases in distal permeability only. For example, the decreased exit of H_2O distally or the increased entry and exit proximally. Retrospective consideration suggested that there might have been complications of this technique which made interpretations of the results difficult. For example, the hypertonic mannitol solutions undoubtedly caused endogenous ADH secretion and thus the exogenous ADH may have caused non physiological responses; the osmotic diuresis may have washed out the hypertonic medullary solutes which provide the normal driving force for H_2O reabsorption and thus the increased net H_2O reabsorption may have been due to a primary effect of ADH on Na transport as has been observed in other epithelia; and there was a possibility of local cardiovascular effects due to ADH.

Because of the above possibilities, the experimental approaches were altered. The possibility of local cardiovascular effects on kidney function were examined by measuring tissue pressures with needles. The interactions of ADH with other hormones was investigated by standard renal clearance techniques. The biochemistry of ADH action on the kidney was investigated by biochemical and isolated tissue techniques.

2. LOCALIZED TISSUE PRESSURES

Effects of hydrostatic pressure altered by either mechanically imposed pressure heads in vitro or through changes in blood pressure or colloid osmotic pressure in vivo have been observed in many epithelia. Results from experiments utilizing epithelia including frog skin, gut and kidney suggest that increased serosal pressures, usually above a threshold value, cause proportional increases in the secretory fluxes. The direct effects of cardiovascular pressure are frequently complicated by simultaneous changes in

tissue colloid osmotic pressures which would tend to reduce hydrostatic pressure effects on epithelial transport and effects of changes in tissue conductivity through which the forces are exerted.

a. Renal Pressure Conductance

In order to evaluate how far pressures could be transmitted through the kidney tissue, needles (25 ga, 2.5 cm) were positioned through a plexiglass guide 5 mm into a kidney exteriorized and immobilized in a plastic holder. The needle guide allowed a relatively precise alignment and known distances between the needle tips. Pressure (15-70 mmHg) was imposed through one of the needles by a mercury manometer and changes in pressure transmitted to the needles at varied distances (0-5 mm) from the pressure source were measured with pressure transducers. Tissue pressures before and during the imposed pressure were measured before and after volume expansion with saline (1 ml/kg·min). The effects of carotid clamping in order to raise blood pressure were also measured before and after volume expansion in order to determine both the effects of changes in blood pressure and the response time of the needles.

Clamping the carotid arteries raised mean blood pressure by an average of 30 mmHg and caused a prompt rise (10 sec) in needle pressure of from 2-10 mmHg with the larger changes usually occurring from lower initial tissue pressures (Fig. 2). All needles in any one animal responded similarly and also there was little difference in the effect of carotid clamping before or after volume expansion.

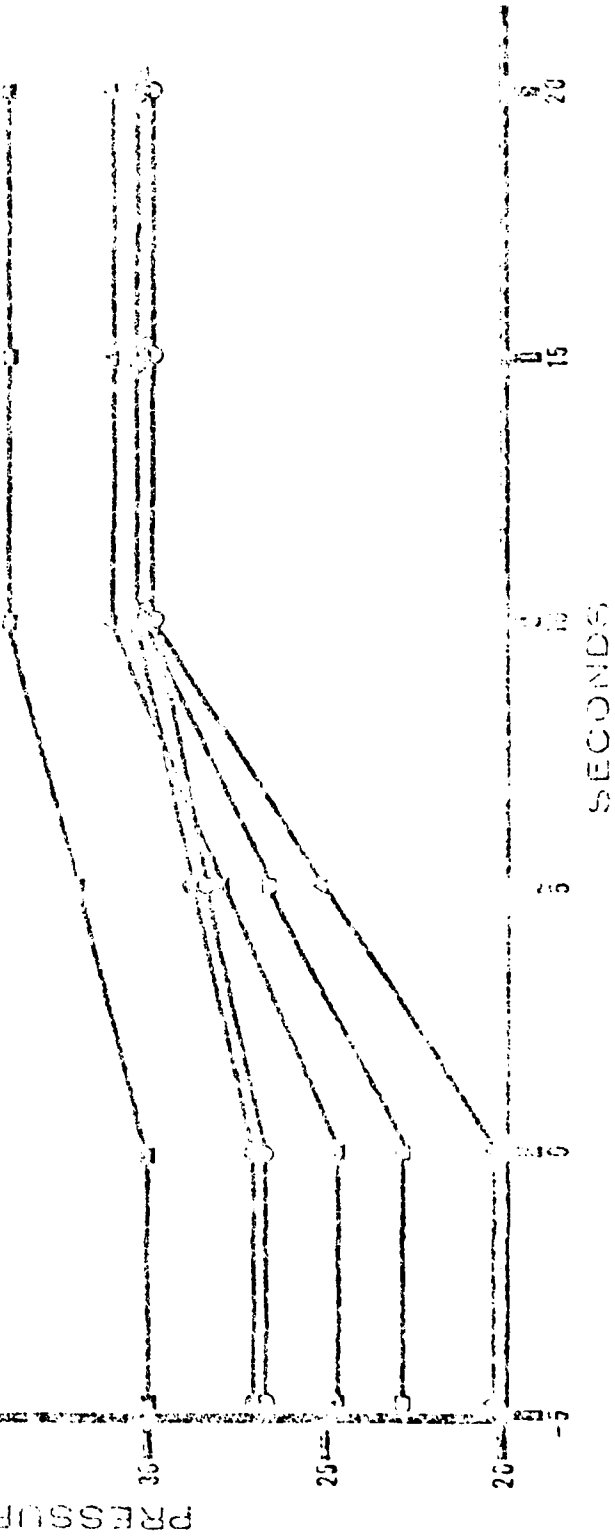
A hydrostatic pressure imposed through one needle was not transmitted more than 2 mm from the source. At a nominal distance of 0 mm, transmitted pressures varied between 30-100% of the imposed pressure and at 1 mm distance transmitted pressure was 0-25% of the imposed pressure (Fig. 3).

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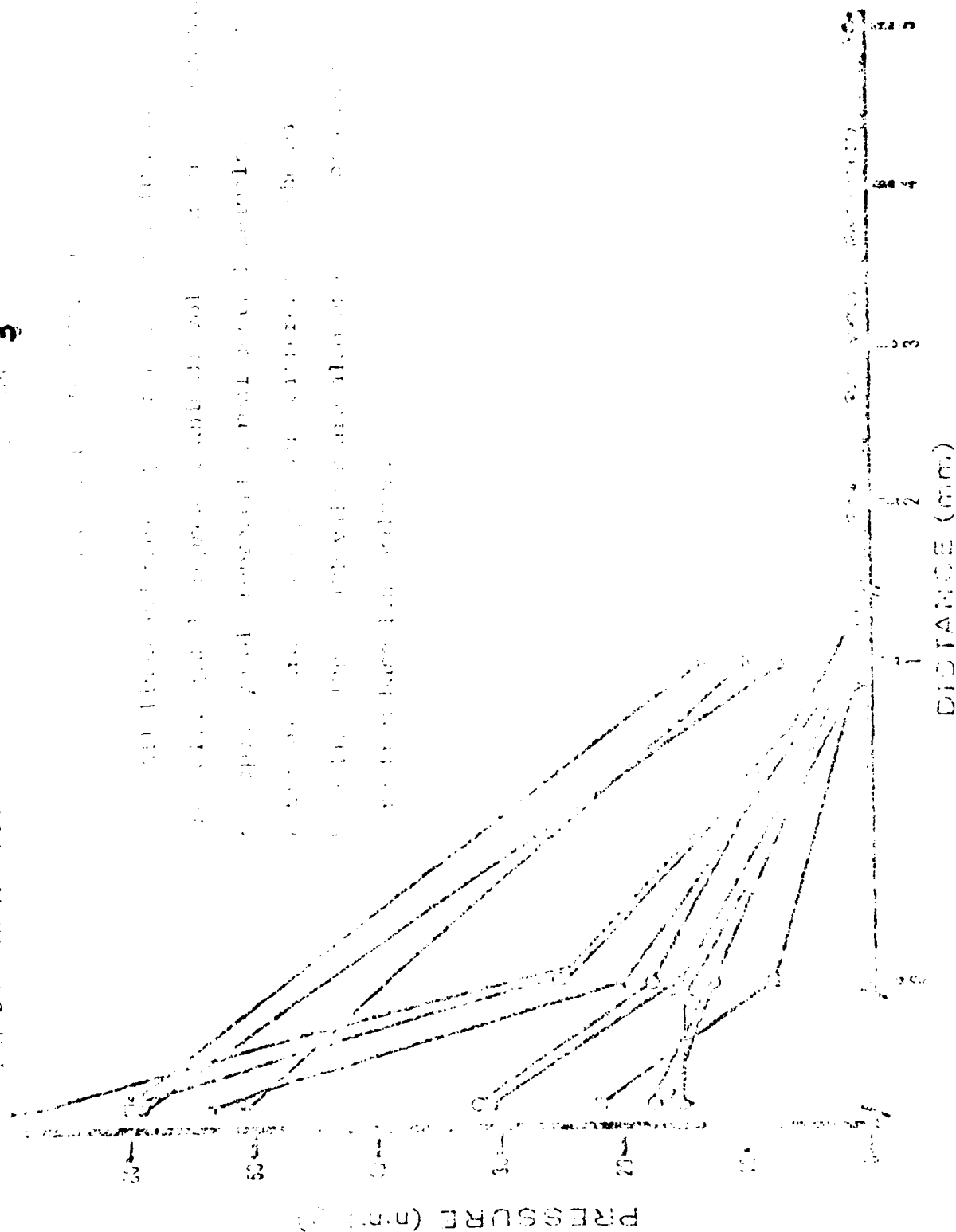
showing that the hypothesis is not supported by the data. The hypothesis is that the probability of a subject making a correct response is greater than the probability of a subject making an incorrect response. The results show that the probability of a subject making a correct response is not significantly greater than the probability of a subject making an incorrect response. Therefore, the hypothesis is not supported by the data.



Changes in AWP with Distance

HYDROSTATIC PRESSURE

3



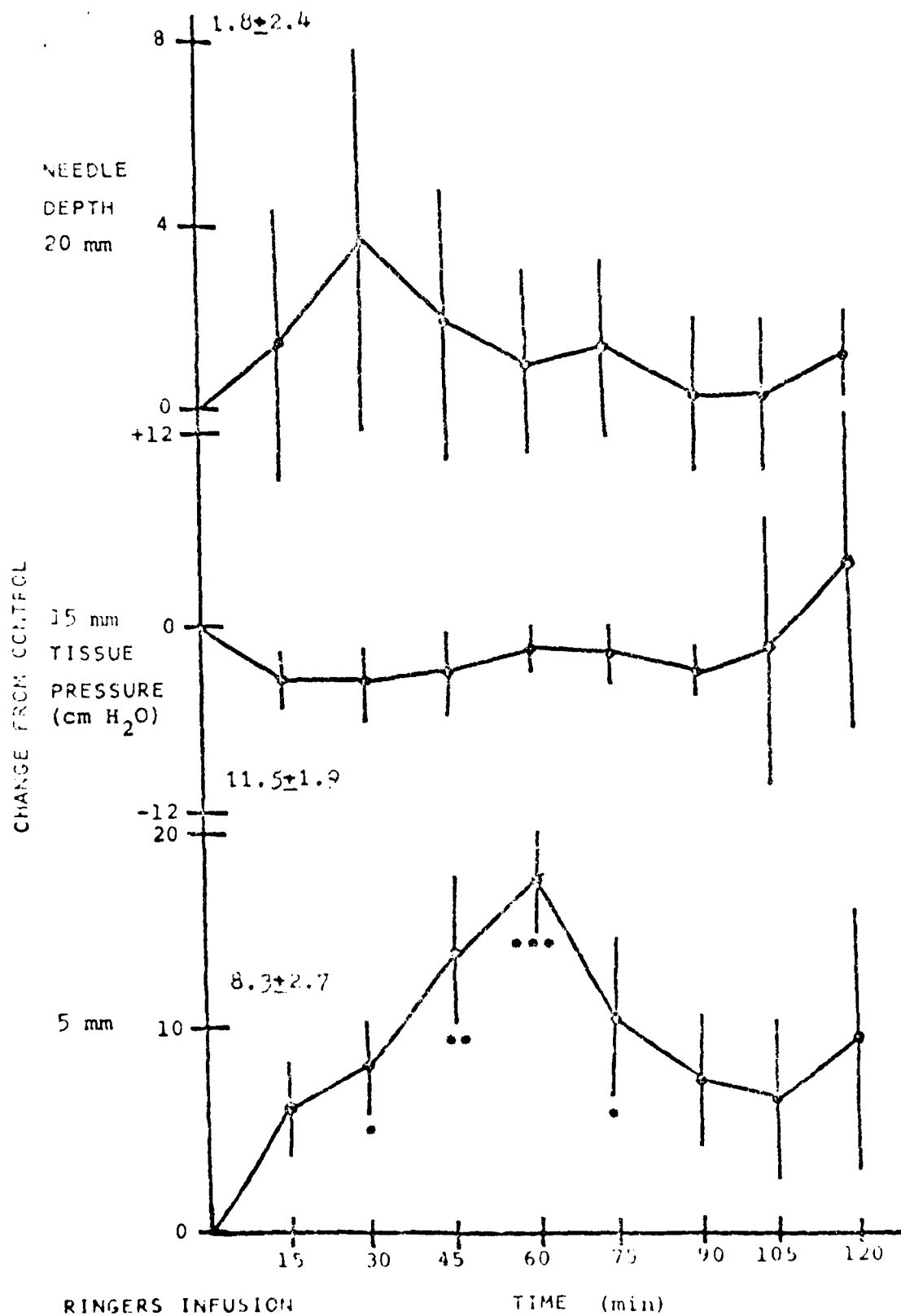
Again there seems to be little difference in pressure transmission before or after volume expansion with saline. Pressure transmission was completed within 0-10 min (data not shown) with the needles at 0 mm distance equilibrating faster than those at 1 mm distance. Saline infusion caused an average increase of tissue pressure of 11.5 ± 5.0 mmHg. The precise distance between needle tips could not be evaluated accurately. Histological examination of the kidneys showed tissue damage around the needle track but the difference between the nominal and actual distance of the needle tips was no greater than 0.5 mm.

The above experiments probably greatly overestimate the degree of pressure transmission in renal tissue because of tissue damage and the imposed pressures opening up channels in the tissue. It is clear that pressures can be restricted to within 2 mm of the source. Thus, localized tissue pressures may occur in the kidney due to local cardiovascular changes. Similar effects probably hold true in other organs.

b. Localized Kidney Tissue Pressure Changes

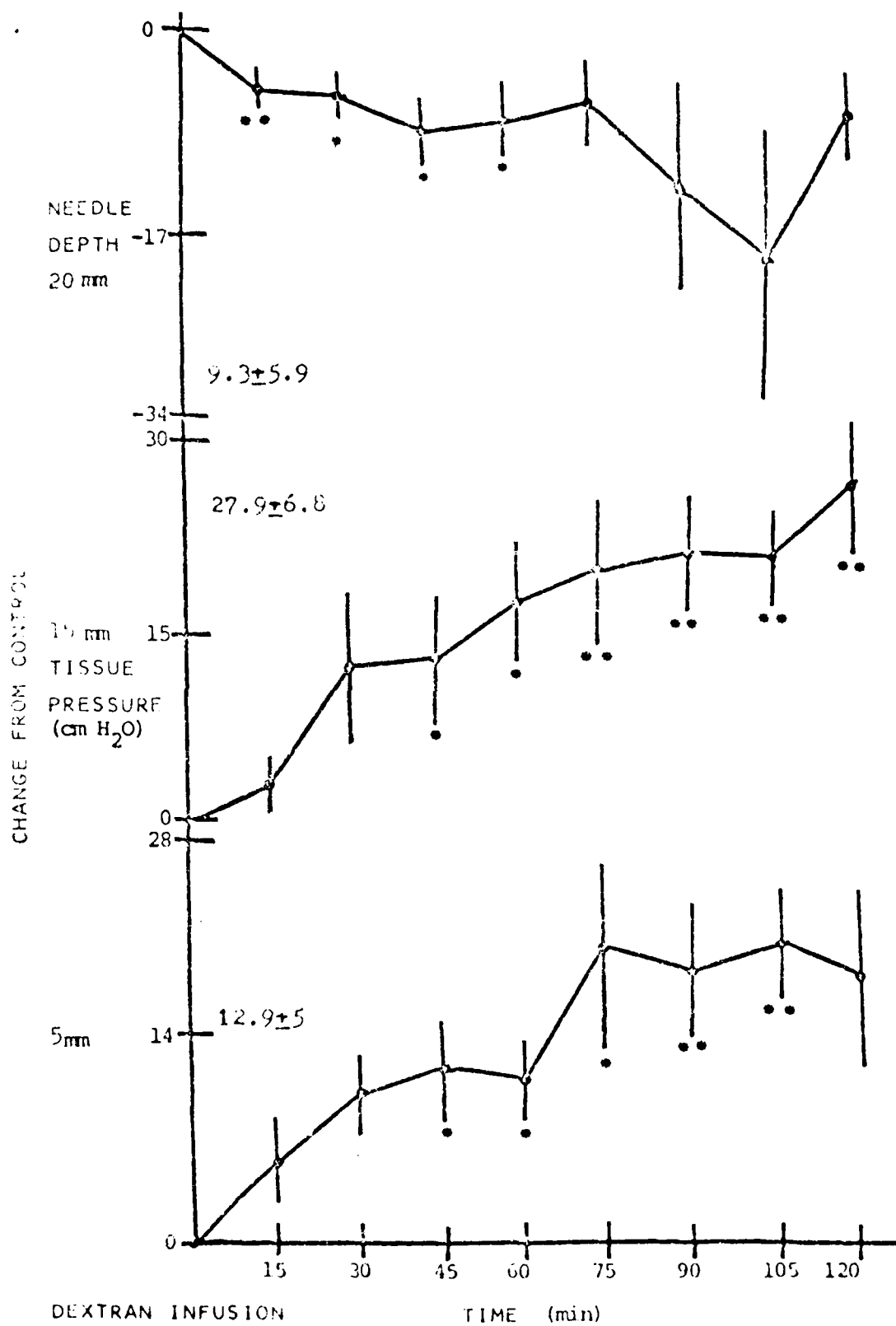
Tissue pressures were measured at different depths in the kidney before and during volume expansion with Ringer solution or 5% dextran (1 ml min⁻¹ kg) or during an osmotic diuresis with 1% mannitol followed by ureteral occlusion. Tissue pressures were measured with needles analogous to the manner described above at depths of 5 mm (cortex), 15 mm (outer medulla) and 20 mm (inner medulla). Ringer infusion caused significant increases in cortical tissue pressure, nonsignificant increases in outer medulla and nonsignificant decreases in inner medulla tissue pressure (Fig. 4). Dextran infusion caused significant increases in tissue pressure in the cortex and inner medulla but significant decreases in the outer medulla (Fig. 5). Mannitol infusion had little effect on cortical or outer medulla tissue pressure

Figure 4. - Effect of Ringer infusion (1 ml/min/kg body weight) on tissue pressures at depths of 5, 15, and 20 mm within the canine kidney (n=7). The Ringer infusion was started after a 60 min control period. Values were compared with control values (at side) using a paired t-test. Values are the mean \pm SEM. *, **, *** represent differences significant at $P < .05$, .01, and .001, respectively.



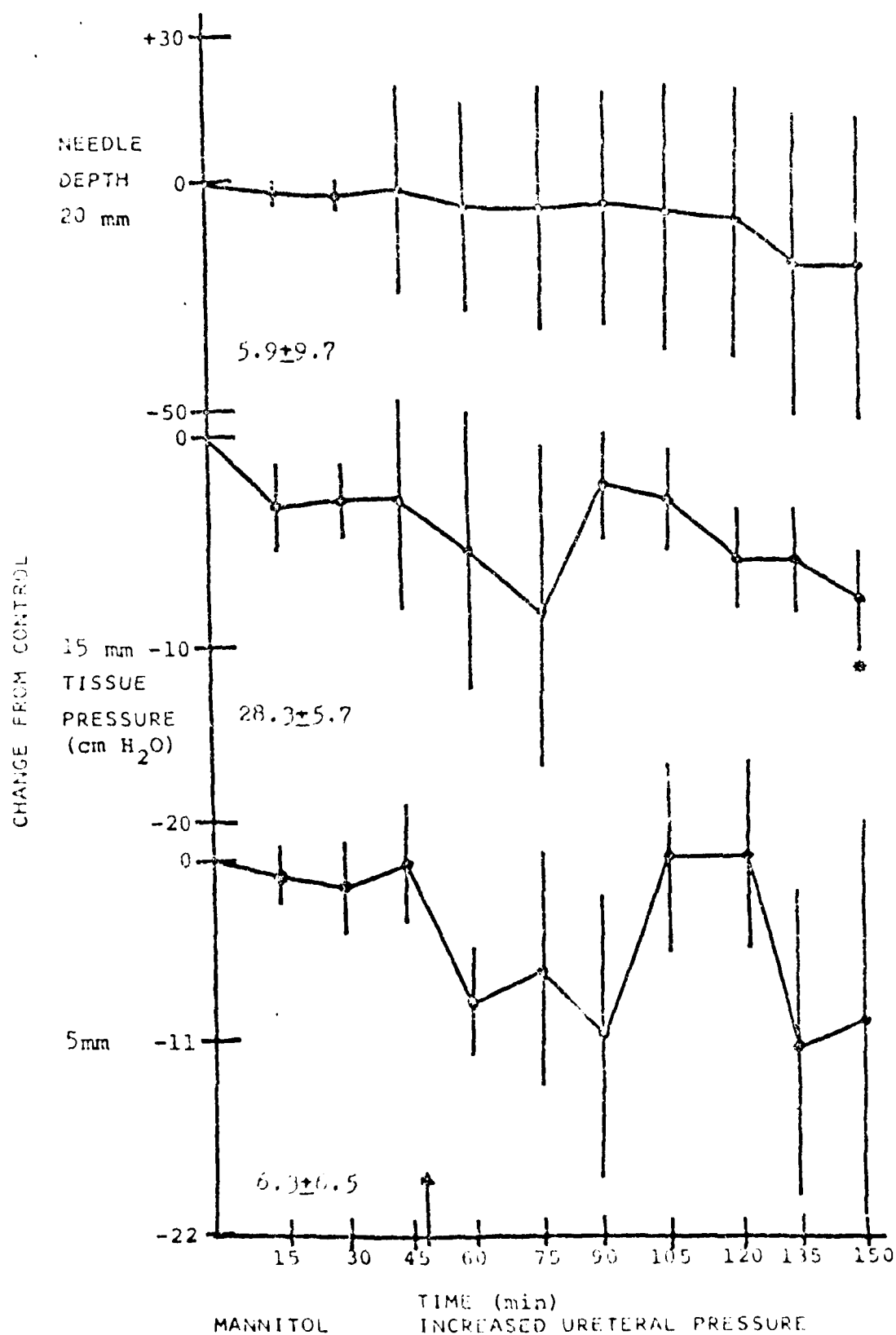
9 b.

Figure 8. - Effect of dextran (5%) (1 ml/min/kg body weight) infusion on tissue pressures at 5, 15, and 20 mm depths within the canine kidney (n=8). The dextran infusion was started after a 60 min control period. Values were compared with control values (at side) using a paired t-test. Values are the mean \pm SEM. *, ** represent differences significant at $P < .05$ and $.01$, respectively.



9 d.

Figure 6. - Effect of mannitol (1%) infusion followed by increased ureteral pressure on tissue pressures at depths of 5, 15, and 20 mm within the canine kidney (1% Mannitol (1%) was started after a 30 min control period. Ureteral pressure was increased after 45 min of mannitol (1%) infusion. Values were compared with control values (at side) using a paired t-test. Values are the mean \pm SEM. * represents differences significant at $P < .05$.



but there were nonsignificant decreases in inner medulla tissue pressures (Fig. 6).

Renal function measurements of urine flow, GFR etc. in the above experiments did not show any simple correlations which could be related to changes in local tissue pressure. For example, changes in renal functions were qualitatively similar for Ringer and dextran infusion although urine flow and Na excretion were much greater with Ringer infusion and yet the changes in inner and outer medulla tissue pressure were generally opposite in the two groups. For the first 60 min, cortical pressures increased in both groups but fell after that time with Ringer infusion but continued to increase with dextran infusion and no value of renal function showed a change in slope at that time in either group.

Therefore, there are localized changes in tissue pressure within the kidney following generalized changes in the cardiovascular system. A direct correlation between these tissue pressure changes and renal function is not obvious probably because of the complexity of the renal system. For example, several different portions of the nephron are within the same level of the kidney and thus would be exposed to the same tissue pressures. Also, simultaneous changes in hormone levels and colloid osmotic pressure could modify effects of tissue pressure changes.

c. Cardiovascular Effects on Renal Function and Subcapsular Pressure

In order to determine if there were a relationship between renal urine formation and cardiovascular effects which could be manifest in tissue pressure changes, subcapsular renal pressures were measured before and during manipulations of blood flow and pressure.

The technique employed in these experiments were standard clearance measurements. Subcapsular pressures were measured through polyethylene

cannules (PE 50) threaded beneath the capsule through a small incision. Subcapsular pressure will reflect cortical pressure. Experimental manipulations were superimposed on the following procedure. Period 1 was a control period; in period 2 arterial blood pressure was increased by carotid clamping; period 3 was a second control and in period 4 the blood pressure of the renal arteries was lowered by a clamp on the aorta. The above procedure was also carried out after a) raising ureteral pressure to 20 mmHg; b) IV saline infusion (10% body weight), c) vena cava occlusion to raise renal vein pressure to 40 mmHg and d) intra-renal artery infusion of the vasodilator bradykinin (5 μ G/min).

The key findings in these experiments were that subcapsular pressure was decreased by ureteral occlusion and increased by bradykinin infusion, venous occlusion and saline infusion in that order. Net Na excretion paralleled subcapsular pressure as did urine flow except during venous occlusion during which manipulation urine volume remained low. Subcapsular pressure was not greatly affected by altering arterial pressure except during bradykinin infusion.

The results suggested that certain experimental manipulations could increase urinary excretion concomitant with cortical tissue pressure. Na excretion usually, but not necessarily, paralleled H_2O excretion. Altering arterial pressure had little effect on subcapsular pressure or urinary excretion unless the renal vasculature had been vasodilated. These results were consistent with an effect of cortical tissue pressure on nephron absorption such that increased tissue pressure reduced nephron reabsorption and thus increased excretion. However, the experiments described below suggest that factors other than tissue pressure per se are more important.

3. ADH EFFECTS ON RENAL MEDULLARY CELLS

The initial step in the activation of cell processes by peptide hormones is the binding of the hormone to an external cell membrane receptor site. The rate of formation of intracellular cyclic AMP is modulated and in turn cyclic AMP affects certain intracellular processes through, in part, cyclic AMP-sensitive protein kinases. ADH is known to modify distal nephron H_2O permeability only at the serosal surface by increasing mucosal H_2O permeability. The cellular basis of these effects are unknown but microtubules and microfilaments have been implicated. Selected aspects of renal medullary membrane function were studied in addition to H_2O permeability responses of medullary tissue slices.

Canine inner medullary tissue slices were preincubated in Ringer solution for 30 min and then distributed into plastic beakers in 20 ml of Ringer solution. After 5-10 min further incubation 0.5 ml of Ringer solution containing $^3\text{H}_2\text{O}$, ^{14}C -inulin and ^{22}Na with or without ADH was added and the uptake of ^{22}Na and $^3\text{H}_2\text{O}$ in tissue slices with added ADH was determined relative to control tissue slices without ADH. Tissue slices were removed at intervals, placed in tared vials, and the uptake of isotope measured by liquid scintillation counting. The incubation fluid was analyzed in a similar manner. Extracellular fluid in the tissue slices was calculated from the tissue content of ^{14}C -inulin and the amount of extracellular $^3\text{H}_2\text{O}$, ^{22}Na and volume subtracted from the total $^3\text{H}_2\text{O}$, ^{22}Na and volume to obtain the intracellular quantities. Cyclic AMP in the tissue slices was determined by radioimmunoassay.

ADH increased cyclic AMP content at a concentration of 10^{-10} M, reached a half maximal response at about 10^{-6} M and a peak effect at about 10^{-5} M ADH. The increase in cyclic AMP was biphasic, increased at 15 sec then

decreasing at 30 sec and increasing again and reaching a constant value at 1.25 min. Relative ^{22}Na uptake was increased by ADH at 10^{-5} and 10^{-7} M but not at lower concentrations. Relative $^3\text{H}_2\text{O}$ uptake was increased by ADH at 10^{-7} and 10^{-8} M but not at higher or lower concentrations. The relative uptake of $^3\text{H}_2\text{O}$ but not ^{22}Na was increased by 10^{-8} M ADH beginning 2.5 min after ADH. In hypertonic Ringer solution ADH (10^{-6} M) caused only a transient increase in cyclic AMP with a peak at 0.75 min. Relative $^3\text{H}_2\text{O}$ uptake, but not ^{22}Na , increased at 0.5 min after ADH (10^{-8} M) when tissue slices were incubated in hypertonic Ringer solution. In the presence of Ca^{++} (5 mM), ADH increased neither cyclic AMP nor $^3\text{H}_2\text{O}$ uptake. Colchicine (10^{-5} M for 30 min), used to disrupt microtubules, did not affect the increase in cyclic AMP but inhibited the increased uptake of $^3\text{H}_2\text{O}$. Ouabain inhibited the increased uptake of $^3\text{H}_2\text{O}$ in response to ADH. Dibutyryl cyclic AMP (10 mM) mimicked the effects of ADH to increase $^3\text{H}_2\text{O}$ and also increased ^{22}Na uptake and these effects were also blocked by colchicine.

The results from these experiments indicated that ADH could change $^3\text{H}_2\text{O}$ uptake in tissue slices in manner analogous to a change in osmotic conductance in vivo. The time course of cyclic AMP increases were consistent with it being the stimulus for the increased $^3\text{H}_2\text{O}$ uptake. ^{22}Na uptake and $^3\text{H}_2\text{O}$ uptake were increased at different ADH concentrations suggesting two separate mechanisms with different cyclic AMP sensitivities. Microtubules were necessary for the increased $^3\text{H}_2\text{O}$ uptake in response to cyclic AMP. Increased Ca^{++} could block the response to ADH probably by blocking the binding of ADH to its receptor sites.

Further studies were carried out on membrane preparations from renal medullary slices. Apical and basilar membranes were purified about 30-fold relative to the initial cell homogenate by sequential density gradient centri-

fugation and free flow electrophoresis. Marker enzymes were Na-K activated ATPase, ADH sensitive adenylate cyclase and ^3H -ADH binding for basolateral membranes and HCO_3^- -ATPase for apical membranes. The apical membranes contained a membrane bound, cyclic AMP sensitive protein kinase, an endogenous substrate and a phosphoprotein phosphatase. The substrate had a molecular weight of 46,000 as determined by SDS gel electrophoresis and scintillation counting of ^{32}P . Basolateral membranes were enriched in cholesterol relative to apical membranes but both had similar contents of phospholipids, sialic acid and hexosamine.

These findings are consistent with the topological separation of ADH binding sites at the mucosal membrane and effector sites involving phosphorylated protein at the apical membrane.

4. CARDIOVASCULAR STUDIES

Changes in blood flow to a transporting organ can affect transport through changes in O_2 delivery to the absorptive cells and also by a washout effect. In this series of experiments, the effects of vasoactive drugs, hormones and neurotransmitters on blood flow and O_2 consumption of the gut were determined. The effects on blood flow and O_2 consumption could not be assigned to specific layers of the gut.

The basic experimental technique was similar for all the experiments. A segment of small intestine was autoperfused through a cannulated mesenteric artery. Blood from the mesenteric vein was returned to the circulation. The segments were perfused at constant flow by means of a pump or by constant pressure through the animals femoral artery and blood flow measured by means of an electromagnetic flow meter. O_2 consumption was determined by measuring the arterial-venous O_2 concentration difference and multiplying it

by blood flow. The permeability-surface area product (PS), equivalent to capillary clearance, was determined from the extraction of ^{86}Rb and blood flow. Colloid reflexion coefficients, a measure of capillary colloid impermeability, was determined from the protein concentrations in plasma and lymph collected from a cannulated mesenteric lymphatic. Labelled adenine nucleosides and nucleotides were determined by thin layer chromatography and liquid scintillation counting.

a. Drug and Neurotransmitter Studies

Ouabain, infused intraarterially, reduced blood flow, O_2 consumption and PS product. Norepinephrine, sympathetic stimulation, ADH and epinephrine at high doses also increased vascular resistance and decreased O_2 consumption and PS product. The catecholamines acted through an alpha-adrenergic mechanism. In contrast epinephrine at low doses increased O_2 consumption, blood flow and PS product through a beta-adrenergic effect. Dopamine decreased gut blood flow, O_2 consumption and PS product which were converted to the opposite responses after blockade of dopamine receptors with haloperidol. The effect of dopamine to increase O_2 consumption, blood flow and PS product were mediated through beta-adrenergic stimulation of receptors which were unmasked by dopamine receptor blockade.

b. Cyclic AMP Studies

Cyclic AMP infused IV or IA is a vasodilator in the mesenteric vasculature. Certain anomalies in the vasodilator action of dibutyryl cyclic AMP (DBCAMP), a widely used analogue of cyclic AMP (CAMP), prompted an investigation into the mode of action of infused cyclic AMP on the mesenteric vasculature.

The effects of CAMP, DBCAMP, AMP, ADP, ATP and adenosine on the mesenteric vasculature were determined. The extracellular and intracellular

metabolism of labelled CAMP and DBCAMP in canine mesenteric arteries was determined utilizing thin layer chromatographic separation of metabolites.

DBCAMP caused a slow (12 min) but ultimately greater (62%) increase in mesenteric blood flow than the quicker (1 min) but smaller increase (41%) in blood flow due to CAMP. ATP, AMP and adenosine caused more rapid and greater increases in blood flow than either CAMP or DBCAMP. DBCAMP was converted to mono butyryl CAMP (MBCAMP) primarily intracellularly. CAMP was mainly degraded to noncyclic nucleotides or nucleosides extracellularly. There was no evidence for the uptake of CAMP into mesenteric arteries. MBCAMP was much more effective than DBCAMP in binding to CAMP receptor sites.

It was concluded that the effects of IV or IA infusions of CAMP are due to extracellular breakdown to adenosine compounds which are the active vasodilators. DBCAMP is slowly taken up by vascular smooth muscle and converted to MBCAMP which is the active vasodilator.

c. Intrinsic Colonic Vascular Responses

The intestine and other organs respond to changes in blood flow and pressure through intrinsic mechanisms which tend to decrease blood flow resistance when blood flow decreases and to increase resistance when pressure increases. The changes in resistance are associated with changes in capillary surface area in some organs which in turn affects the rate of total capillary ultrafiltration. These phenomena have been extensively investigated in the small intestine but little work has been done on the colon.

The blood flow at the intestinal absorptive site (ASBF) was measured in the colon as the clearance of $^3\text{H}_2\text{O}$ as described later in this report for the small intestine. Total blood flow was measured by an electromagnetic flow meter. Colonic capillary colloid permeability was calculated from lymph protein

concentration during increased lymph flow. Vascular responses were induced by decreasing arterial pressure, increasing venous pressure or IA infusion of adenosine.

ASBF was found to be strongly autoregulated in response to either arterial pressure decreases or venous pressure increases i.e., ASBF remained constant at arterial pressures between 20-140 mmHg and venous pressures between 5-35 mmHg. Total blood flow decreased markedly during these same procedures. The constancy of ASBF allowed colonic water absorption to remain constant at arterial blood pressures between 10-140 mmHg. Net H_2O absorption was constant as arterial pressure decreased due to decreases in the unidirectional absorptive and secretory fluxes. Increasing venous pressures caused increased lymph flow associated with decreased lymph protein concentration. Lymph protein concentration reached a constant low value where upon it became independent of lymph flow rate. The final lymph: plasma protein concentration was 0.15 indicating that colonic capillaries, even though they are fenestrated, severely restrict colloid movement and thus allow about 35% of the theoretical colloid osmotic pressure to be exerted across the capillaries. Adenosine, a metabolite implicated as a normal regulator of blood flow responses to tissue activity, increased both total blood flow and ASBF but did not alter net H_2O absorption because of equal increases in the absorptive and secretory H_2O fluxes.

It was concluded that one of the major homeostatic mechanisms in the colon is to maintain a constant ASBF in order to maintain net H_2O absorption constant. The relatively colloid-impermeable colonic capillaries provide a significant driving force for the uptake of interstitial fluid. Probably, changes in Starling forces, capillary and tissue hydrostatic and colloid osmotic pressure, and ASBF are altered in a manner which prevents disproportionate

changes in the secretory unidirectional H_2O flux as compared to the absorptive H_2O flux. Adenosine has effects which are consistent with it being a major regulatory metabolite in the control of colonic ASBF.

5. INTESTINAL ABSORPTION AND HEMODYNAMICS

Several of the studies on kidney function as well as the literature suggested that local hemodynamics could alter renal epithelial transport. The complexity of the kidney anatomy and the different tissue pressures in different regions of the kidney, made a correlation between hemodynamics and nephron function difficult. For this reason, as well as intrinsic interest because of its contribution to salt and water metabolism, the regulation of intestinal absorption of salt and water was studied.

In order to relate local hemodynamic changes to intestinal absorption the blood flow at the absorptive site adjacent to the epithelium had to be estimated. Absorptive site blood flow (ASBF) was estimated by the clearance of $^3\text{H}_2\text{O}$ from the lumen after validation experiments suggested that the diffusion of $^3\text{H}_2\text{O}$ from the lumen into the ASBF was so rapid that the $^3\text{H}_2\text{O}$ concentration in the venous drainage from the absorptive site was nearly equal to that in the lumen. ASBF could then be calculated as $^3\text{H}_2\text{O}_{\text{ABS}} / [^3\text{H}_2\text{O}]_{\text{L}} - [^3\text{H}_2\text{O}]_{\text{A}}$ where $^3\text{H}_2\text{O}_{\text{ABS}}$ represents the total amount of $^3\text{H}_2\text{O}$ absorbed from the lumen, $[^3\text{H}_2\text{O}]$ represents $^3\text{H}_2\text{O}$ concentration and L and A represent lumen effluent and mesenteric artery.

The following studies utilized essentially the same technique. A segment of terminal epithelium with its nerve and blood supply intact was perfused through the lumen with saline containing $^3\text{H}_2\text{O}$, ^{14}C -inulin, and ^{22}Na . A mesenteric vein draining the segment was cannulated for obtaining venous plasma samples and blood pressure. Drug or hormone infusions were into a

femoral vein or a mesenteric artery to the perfused gut segment. Net and unidirectional Na and H₂O fluxes were calculated by the method of Berger & Steele.

The main purposes of these studies was to distinguish between effects of the experimental manipulations on transcellular carrier-mediated transport from those exerted through effects on passive, paracellular transport through the tight junctions and lateral intercellular spaces. Based on theoretical considerations and the literature as well as initial findings the results were tentatively interpreted in the following manner. Changes in ABSF which were linearly correlated with absorptive H₂O fluxes along the same line as in controls were interpreted as due to a "washout" effect of blood flow on absorption. Similarly, secretory fluxes which correlated with estimated capillary pressure along the same line as in controls were considered to represent changes in passive movement due to ultrafiltration. Deviations in slope or intercept as compared to controls suggested a change in epithelial transport.

a. Body Fluid Expansion

Intravenous infusion of either saline or saline followed by hyperoncotic dextran in saline caused changes in Na and H₂O fluxes which were completely consistent with effects exerted through the cardiovascular system. ABSF was unchanged but venous pressure increased and colloid osmotic pressure decreased and net absorption was converted to net secretion due almost solely to an increase in the unidirectional secretory fluxes. Hyperoncotic dextran transiently reversed the changes due to saline. These effects were independent of the Na concentration in the lumen.

b. Glucagon

Intrararterial infusion of glucagon into the perfused gut segment or into a non-perfused adjacent gut segment caused generally opposite changes in gut

absorption and ASBF. These were interpreted as due to the direct vasodilator action of glucagon being antagonized by reflex sympathetic vasoconstriction initiated by a decrease in systemic blood pressure. Na and H_2O fluxes both absorptive and secretory were directly correlated with estimated capillary pressure. Changes in ASBF shifted the relationship to a different linear function. The effects of glucagon could not be attributed to the concomitant increase in blood glucose or insulin nor were they mimicked by the vasodilator histamine. The conclusion that glucagon changed gut absorption by a cardiovascular effect was also suggested by subsequent experiments by other investigators.

c. Vasoactive Intestinal Peptide (VIP)

Intravenous VIP caused intestinal secretion. In contrast to the direct proportionality between secretory fluxes and estimated capillary pressure observed with saline infusion and glucagon, there was a negative correlation during VIP infusion. This was interpreted as an indication that a secretory transcellular pump was overriding the simultaneous tendency for reduced capillary pressure to reduce secretory fluxes. Absorptive fluxes were positively correlated to ASBF as observed in subsequent experiments. There were major involvements of the parasympathetic autonomic nervous system in the effects of VIP because atropine blocked most of the effects of VIP.

d. Pentagastrin and Gastrin

Pentagastrin (the C-terminal pentapeptide of gastrin) has qualitatively similar effects compared to gastrin on several physiological functions e.g. gastric H^+ secretion. However, pentagastrin (G5) had effects different than those of gastrin (G17) on intestinal absorption. G5 decreased intestinal absorption in both fed and fasted dogs and G17 increased absorption. ASBF was positively correlated with the unidirectional absorptive fluxes but the

unidirectional secretory fluxes were generally inversely correlated with estimated capillary pressure. As with VIP, the negative correlation suggested that a secretory pump had been stimulated by pentagastrin. Further studies showed that after atropine pretreatment, both G5 and G17 (at lower doses) increased gut absorption but after guanethidine pretreatment G5 caused net secretion. This finding suggested that the main effect of G5 was to stimulate the release of acetylcholine which in turn reduced gut absorption and that G17 was less effective in releasing acetylcholine but was more effective than G5 in stimulating absorption.

e. Morphine

Morphine or other opiates are widely used as anti-diarrheal compounds and endogenous opiates are found in the gut. Morphine was infused IV into fed and fasted dogs. Morphine increased intestinal absorption in fed but not in fasted dogs due to an increase in absorptive fluxes associated with an increase in ASBF. Secretory fluxes were reduced relative to estimated capillary pressure in fed dogs as compared to fasted dogs. The increased absorption in fed dogs but not in fasted dogs suggests that morphine may act by modifying another process stimulated by feeding. The major effect of morphine could be exerted through a primary effect on blood flow and increased washout or through an effect to increase transcellular transport with a consequent increase in O_2 consumption and a secondary increase in blood flow.

f. Ultrastructural Changes

During capillary ultrafiltration or absorption the increased entry of fluid into the interstitial space increases its hydraulic conductance and thus allows easier fluid movement between the blood and lumen. Ultrastructural changes in epithelia of gall bladder and nephron have been observed during changes

in net fluid absorption. The possibility of ultrastructural changes in the small intestine during absorption with superimposed volume expansion was investigated along with concurrent changes in fluxes.

Rat jejunum was perfused with Ringer solution containing $^3\text{H}_2\text{O}$, ^{14}C -inulin and ^{22}Na . After control fluxes were measured, the rat was volume expanded with Ringer solution with a volume of 5% or 10% of body weight over 10 min. After a 10 min equilibration period, unidirectional and net fluxes were again determined. Control animals had fluxes measured over the same time period but were not volume expanded. Segments of perfused intestine and non-perfused adjacent intestine were quickly removed and placed in 3% Karnovsky's fixative +0.2 M s-collidine (4°C), the lumen perfused with fixative, rinsed in collidine after 2 hrs, postfixed in 1% OsO_4 -collidine, dehydrated and embedded in Spurr's plastic. Tissue sections were mounted on grids and stained in uranyl acetate and Reynold's lead citrate. Sections were examined with a Hitachi HS-8 electron microscope at 50 KV. Dimensions of structures were determined with an ocular micrometer on randomly selected photomicrographs.

Absorption was associated with expansion of the lateral intercellular spaces. Volume expansion caused a collapse of the lateral spaces and the appearance of "blisters" in the tight junctions at 5% volume expansion and further expansion of the lateral intercellular spaces at 10% volume expansion. There were no significant changes in the degree of folding of the lateral spaces as judged by a constant ratio of lateral membrane length per cell length. The diameter of the rough endoplasmic reticulum increased in proportion to volume expansion but was smaller in the perfused gut segments as compared to non-perfused gut segments. Net Na absorption decreased significantly after 5% volume expansion due to significant increases in the

secretory Na and H₂O fluxes. Net Na and H₂O absorption were not significantly changed following 10% volume expansion due to increases in both the secretory and absorptive fluxes. ASBF was significantly increased only after 10% volume expansion.

It was difficult to correlate many of the ultrastructural changes with the changes in fluxes. The increased secretory fluxes following 5% volume expansion were associated with "blisters" in the tight junction and collapse of the lateral spaces expanded by absorption. Possibly, disruption of the tight junctions allowed fluid movement out of the lateral spaces thus relieving interstitial pressure. This possibility is in agreement with the findings of others that lymph flow decreased at the same time that secretion increased during volume expansion. The disappearance of the blisters concurrently with the further enlargement of the lateral space after 10% expansion may be due to a larger increase in interstitial pressure which cannot be compensated by leakage through the tight junctions. The expanded lateral spaces may also increase the conductance thus allowing the greater absorptive fluxes. The dilated endoplasmic reticulum seems more closely correlated with volume expansion as such and absorption may open more of the endoplasmic reticulum to the lumen thus reducing the diameter relative to non-pertused gut.

SUMMARY

The results of these experiments are consistent with several hormones altering transcellular transport and in addition modifying net transport through physical effects exerted through paracellular transport. The evidence was better for gut transport than kidney transport because of the greater anatomic complexity of the kidney. The responses of H₂O transport to ADH in the whole kidney could be mimicked in tissue slices and the

responses were consistent with the effector being a cyclic AMP sensitive, apical membrane protein kinase.

SECTION II. DISSERTATIONS AND PUBLICATIONS

Graduate Students Supported by ONR Contract

1. Dr. Robert Dillon - M.S., 1968. "The Relationship Between Cardio-vascular Changes and Intestinal Absorption of Salts and H_2O in the Canine Ileum Following Hemorrhage." Mr. Dillon entered and graduated from medical school.
2. Dr. Roberto San Martin, M.S., 1969. "Effects of Head Upward Tilting on Na and H_2O Fluxes Across the Canine Ileum." Mr. San Martin entered and graduated from medical school.
3. Mr. David Jessel - M.S., 1970. "Renal Permeability Changes Following Antidiuretic Hormone Administration in Canis familiaris." Mr. Jessel started a computer consulting firm.
4. Dr. Thomas Burke - Ph.D., 1970. "Site of Action of Antidiuretic Hormone in the Mammalian Nephron." Dr. Burke did postdoctoral research at Duke University under Dr. R. Robinson and is now on the faculty of the University of Colorado Medical School.
5. Dr. LaNelle Geddes - Ph.D., 1970. "Latency Relaxation in Frog Skeletal Muscle." Dr. Geddes did postdoctoral research with Dr. Baker at Baylor Medical School and later was on the Baylor faculty.
6. Dr. Robert Puddy - Ph.D., 1970. "Ion and carbohydrate Absorption in the Posterior Intestine of Cryptochiton stelleri." Dr. Puddy is presently on a college faculty.
7. Dr. Louis Schneider - Ph.D., 1971 - "Transport Mechanisms in the Anterior Intestine of Cryptochiton stelleri." Dr. Schneider did post-doctoral research with Dr. H. Shedl at the University of Iowa Medical School and remained with him as a Research Associate.
8. Dr. Roger Novak - Ph.D., 1971. "Isolation of the Glucose Carrier Molecule in Red Blood Cells." Dr. Novak did postdoctoral research with Dr. Lefevre at the University of New York, Stony Brook and later was a Research Associate at the University of Texas Medical School at Galveston.
9. Mr. Peter Foster - M.S., 1971. "Effect of Norepinephrine and Anti-diuretic Hormone on Salt and H_2O Excretion by the Kidney." Mr. Foster entered and graduated from medical school.
10. Mr. Kirk Jordan - M.S., 1975. "The Effects of Hydrostatic and Osmotic Pressure on Blood Flow and Unidirectional Fluxes of Sodium and Water in the Canine Ileum." Mr. Jordan is presently doing applied research in food technology.

11. Mr. Samuel MacFerran - M.S., 1975. "The Effects of Glucagon on Sodium and Water Unidirectional fluxes in the Dog Intestine." Mr. MacFerran is on a college faculty.
12. Mr. Kenneth Lepper - M.S., 1976. "A Physiological and Ultrastructural Examination of Rat Jejunum." Mr. Lepper is working with a firm doing medical applications of computers.
13. Dr. S. V. Iyengar - M.S., 1976. "Vasopressin Sensitive H_2O Transport System of Canine Renal Medulla." Dr. Iyengar continued on to the Ph.D. degree.
14. Dr. S. V. Iyengar - Ph.D., 1977. "Biochemical Studies on the Possible Mechanisms of Action of Vasopressin on Canine Renal Medulla." Dr. Iyengar took a postdoctoral appointment with Dr. L. Birnbaumer at Baylor Medical School.

Published Papers:

1. R. San Martin and D. S. Mailman. Effect of Head Upward Tilting on Unidirectional Na and H₂O Fluxes Across the Canine Ileum. *Proc. Soc. Exp. Biol. Med.* 140, 694-699, 1972.
2. Lawrence, A. L., Mailman, D. S. and Puddy, R. L. The Effect of Carbohydrates on the Intestinal Potentials of *Cryptochiton stelleri*. *J. Physiol.* 225, 515-528, 1972.
3. L. Geddes and D. S. Mailman. Contrasting Twitch and Latency Relaxation in Skeletal Muscle. *Comp. Biochem. and Physiol.* 44, 693-710, 1973.
4. Shepherd, A. P., Mailman, D. S., Burks, T. F. and Granger, H. J. Sympathetic Nervous Control of Intestinal Extraction in Oxygen Transport to Tissues. *Plenum Press.* 423-428, 1973.
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13. Lepper, K. and Mailman, D. An Ultrastructural Examination of Everted Rat Jejunum. *Life Sci.* 20, 1697-1704, 1977.

14. MacFerran, S. and Mailman, D. Effects of Glucagon on Canine Intestinal Sodium and Water Fluxes and Regional Blood Flow. *J. Physiol.* 266, 1-12, 1977.
15. Mailman, D. Fluid and Electrolyte Transport. *Gastrointestinal Physiology*, ed. Johnson, L. 1st ed. C. V. Mosby Co. 109-121, 1977.
16. Mailman, D. Different Mechanisms of Action of Vasoactive Intestinal Polypeptide (VIP) and Glucagon on Intestinal Secretion. *Gastroenterology* 73, 1463-1465, 1977.
17. Mailman, D. Effects of Vasoactive Intestinal Polypeptide on Intestinal Absorption and Blood Flow. *J. Physiol.* 279, 121-132, 1978.
18. Iyengar, R., Sachs, G. and Mailman, D. Purification of Distinct Plasma Membranes from Canine Renal Medulla. *Amer. J. Physiol.* 234, F247-F254, 1978.
19. Mailman, D. Effects of Pentagastrin on Intestinal Absorption and Blood Flow. *J. Physiol.* 307, 429-442, 1980.
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